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The promoter of the nematode resistance gene Hs1^{pro-1} activates a nematode-responsive and feeding site-specific gene expression in sugar beet (*Beta vulgaris* L.) and *Arabidopsis thaliana*

Tim Thurau, Sirak Kifle, Christian Jung and Daguang Cai*
*Institute of Crop Science and Plant Breeding, Christian Albrechts University of Kiel, Olshausenstrasse 40, 24098 Kiel, Germany (*author for correspondence; e-mail dcai@plantbreeding.uni-kiel.de)*

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Abstract

The Hs1^{pro-1} gene confers resistance to the beet cyst nematode *Heterodera schachtii* in sugar beet (*Beta vulgaris* L.) on the basis of a gene-for-gene relationship. RNA-gel blot analysis revealed that the transcript of Hs1^{pro-1} was present in uninfected roots of resistant beet at low levels but increased by about fourfold one day after nematode infection. Treatments of plants with external stimuli including salicylic acid, jasmonic acid, gibberellic acid and abscisic acid as well as wounding or salt stress did not result in changes in the gene transcription, indicating *de novo* transcription of Hs1^{pro-1} upon nematode infection specifically. To study transcriptional regulation of Hs1^{pro-1} expression at the cellular level, a 3082 bp genomic fragment representing the Hs1^{pro-1} promoter, isolated from the YAC-DNA housing the Hs1^{pro-1} gene, was fused to the β-glucuronidase reporter gene (1832prm1::GUS) and transformed into susceptible beet roots and *Arabidopsis* plants, respectively. Fluorometric and histochemical GUS assays on transgenic beet roots and *Arabidopsis* plants carrying the 1832prm1::GUS construct demonstrated that the Hs1^{pro1} promoter is functional in both species and drives a nematode responsive and feeding site-specific GUS-expression. GUS activity was detected as early as at initiation of the nematode feeding sites and GUS staining was restricted to the nematode feeding sites. To delineate the regulatory domains of the Hs1^{pro-1} promoter, fusion genes with various 5′ deletions of the Hs1^{pro-1} promoter and the GUS gene were constructed and analysed in transgenic beet roots as well. *Cis* elements responsible for feeding site-specific gene expression reside between –355 and +247 from the transcriptional initiation site of Hs1^{pro-1} whereas an enhancer region necessary for higher gene expression is located between −1199 and −705 of the promoter. The Hs1^{pro-1} promoter drives a nematode feeding site-specific GUS expression in both sugar beet and *Arabidopsis* suggesting a conserved mechanism of regulation of Hs1^{pro-1} expression in these two species.

Introduction

Plants have established an innate immune system based on dominant resistance gene (*R* gene). Resistance gene products are considered to be implicated in both recognition of elicitors (ligands) encoded by avirulence (*Avr*) genes and in activation of downstream signalling components. When *R* and *avr* gene products of matched specificity are present and interacted each other in a direct or indirect manner, race-specific resistance responses are induced, which is often associated with the rapid, localized cell death of the hypersensitive responses (HR) that finally restrict pathogen growth or reproduction in plants (Dangl and Jones, 2001).

In recent years, a number of gene-for-gene-type resistance genes have been isolated from a range of dicot and monocot species conferring resistance to a variety of plant pathogens and intensively studied at the genetic and molecular levels. A major class of *R* genes in a diverse range of species encode proteins that contain a variable N-terminal domain, followed by a

putative nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) motifs (reviewed by Baker *et al.*, 1997; Ellis *et al.*, 2000; Dangl and Jones, 2001). The NBS domain is found not only in a large variety of nucleotide-biding proteins with ATP- or GTP-binding function in diverse organisms (Traut, 1994) but also shares high homology with the APAF1 and CED-4 and CARD4 proteins that are involved in animal innate immunity and apoptosis (Saraste *et al.*, 1990, Li *et al.*, 1997; van der Biezen and Jones, 1998, Aravind *et al.*, 1999), suggesting a conserved mechanism of the cell death programmes in plants, animals and mammals as well. Elicitor recognition specificity is believed to be determined by the LRR domain (Ellis *et al.*, 1999) while the N terminus, probably together with NBS domain, participates in activating downstream signal transduction components leading to pathogen-specific resistance responses (Aarts *et al.*, 1998; Feys and Parker, 2000; van der Biezen *et al.*, 2000).

Nevertheless, very little is known about transcriptional regulation of the plant R gene in plant cells. Because most plant disease resistance genes cloned so far have been reported to be expressed constitutively, their regulation at the transcriptional level have been not investigated further. However, studies on a few R genes, such as *Xa1*, *Xa21*, *Pib* and *Pi-ta* in rice and *Rp1-D* in maize, demonstrated that they are transcriptionally regulated either by pathogen infection or by environmental stress, wounding or by treatment with certain chemicals (Yoshimura *et al.*, 1998; Century *et al.*, 1999; Wang *et al.*, 2001), suggesting an important role of transcriptional regulation of R genes in activating disease resistance responses. However, no any study has been reported so far regarding the promoter analysis of R genes.

Nematodes are serious pests in many crops and cause severe economic losses (Sasser, 1987). Economically most relevant are sedentary endoparasites of the genera *Heterodera* and *Globodera* (cyst nematodes) and of the genus *Meloidogyne* (root-knot nematodes). Typically, cyst nematodes induce and maintain multinucleate syncytia in the vascular cylinder of their host plants. This extensive feeding structure, resulted from partial cell wall degradation between cells of the vascular parenchym and the subsequent fusion of the protoplasts (Sobczak *et al.*, 1997), are the only source of nutrients for the developing nematodes. Therefore, they play a central role in both of compatible and incompatible host-pathogen interactions (Böckenhoff *et al.*, 1996; Golinowski *et al.*, 1997; Hussey and Grundler, 1998).

The structure and function of the nematode feeding apparatus and the associated root cell responses to feeding have been comprehensively investigated at the cellular and molecular level (Wyss, 2002). Hatched J2 juveniles invade the roots predominantly in the zone of elongation and then migrate intracellularly through cortical cells towards the vascular cylinder to select the initial cell for syncytium induction (ISC, initial syncytial cell). The first visible changes in the ISC become apparent only a few hours after the J2 have started feeding. It has been observed that the J2 of both cyst nematodes and root knot nematodes synthesize β-1,4-endoglucanases (Egase) in the two subventral glands that are secreted through the stylet (Smant *et al.*, 1998; de Boer *et al.*, 1999; Goellner *et al.*, 2000). These cellulases are thought to soften root cell walls and thus assist intracellular migration. Secretion of these Egases by cyst nematodes into plant root cortical tissues was observed but not into developing syncytia suggesting that the cell wall modifications within feeding sites arise from cell wall-modifying enzymes of plant rather than nematode origin (Goellner *et al.*, 2001).

Cellular and physiological alterations involved in the formation and differentiation of nematode feeding structure require a highly co-ordinated gene expression and regulation (Gheysen *et al.*, 1996; Williamson and Hussey, 1996; Fenoll *et al.*, 1997; Davis *et al.*, 2000). Changes in the expression of specific genes in nematode feeding sites have been documented recently by using molecular techniques (Sijmons *et al.*, 1994; Opperman *et al.*, 1994; Gheysen *et al.*, 1996; Fenoll *et al.*, 1997; Puzio *et al.*, 1999). An enhanced expression of a catalase gene was found after infection by the potato cyst nematode (Niebel *et al.*, 1995). *LEMMI9*, a late-embryogenesis-abundant (LEA)-like gene, and extensin genes were found to be up-regulated in giant cells of tomato plants infected with root knot nematodes (Van der Eycken *et al.*, 1996). Also, the expression of the *pyk20* gene was enhanced in syncytia of *Arabidopsis* roots after infection with the cyst nematode *Heterodera schachtii* (Puzio *et al.*, 1999). Recently it has been demonstrated that the expression of five different tobacco Egase genes are up-regulated in plant roots infected by cyst and root knot nematodes (Goellner *et al.*, 2001). Several reports have been given about promoter sequences triggering down- and up-regulation of genes within feeding sites (Cramer, 1992; Goddijn *et al.*, 1993; Niebel *et al.*, 1996; Barthels *et al.*, 1997; Fenoll *et al.*, 1997; Favery *et al.*, 1998; Puzio *et al.*, 1998; Escobar *et al.*, 1999).

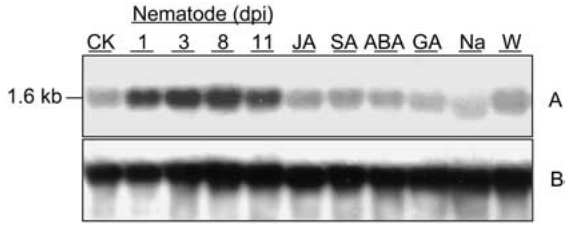


Figure 1. Determination of Hs1^{pro-1} expression level in root tissue of the nematode-resistant sugar beet line A906001 under biotic and abiotic stress as well as after nematode infection by RNA-gel blot analysis. A 20 μg portion of total RNA was loaded in each lane and probed with 1832dh representing 543 bp of the Hs1^{pro-1}-coding sequence (not shown in Figure 2). CK, roots without treatment; Nem. (dpi), roots inoculated with nematode J2 juveniles 1, 3, 8 and 11 days after infection; JA, roots treated with 100 μM jasmonic acid; SA, roots treated with 2 mM salicylic acid; ABA, roots treated with 100 μM abscisic acid; GA, roots treated with 100 μM gibberellic acid; W, roots wounded with a razor blade. Hybridization with an 18S rDNA probe served as a loading control (B). Molecular length markers are indicated at left in kb. Exposure time: 72 h.

All of these studies, taken together, suggest that nematodes induce changes in activity of plant transcription factors that control the formation of syncytia and giant cells (Escobar *et al.*, 1999).

In the past five years, several nematode resistance genes have been cloned from sugar beet, tomato and potato, which confer resistance to nematodes relying on the gene-for-gene relationship (Cai *et al.*, 1997; Milligan *et al.*, 1998; van der Vossen *et al.*, 2000; Ernst *et al.*, 2002). Complete resistance of sugar beet (*Beta vulgaris* L.) to the beet cyst nematode (*H. schachtii* Schm.) is due to the Hs1^{pro-1} gene that has been introduced into cultivated beet from the wild species *B. procumbens* (Jung and Löptien, 1986). The Hs1^{pro-1} gene has been cloned by a map-based cloning strategy (Cai *et al.*, 1997). The predicted protein encoded by Hs1^{pro-1} has a leucine-rich region and a putative transmembrane domain but it does not belong to the NBS-LRR class of plant resistance genes and fit any other existing R proteins. It is therefore attempting to speculate that Hs1^{pro-1} presents a member of an entirely new class of R genes (Jung *et al.*, 1998). As no homology to any gene in public databases has been found so far, the function of the Hs1^{pro-1} protein is still unknown. However, host-pathogen interactions have been studied intensively at the cellular level. Infective juveniles are able to invade the root of resistant plants and proceed to the vascular cylinder to induce formation of syncytia. In contrast to susceptible roots, nematode-feeding structure exhibited strong necrosis and disruption of the feeding cells yielded shortly after nematode infection in the formation of specific membrane aggregations. They were condensed to electron dense bodies filling large parts of the syncytium causing complete degradation of the syncytia and the death of the juveniles (Holtmann *et al.*, 2000).

In this paper, we report on the isolation and characterization of regulatory sequences upstream of the Hs1^{pro-1} gene. The expression pattern of Hs1^{pro-1} was investigated under biotic and abiotic stresses as well as after nematode infection revealing that Hs1^{pro-1} was up-regulated transcriptionally in resistant beet roots upon nematode infection. A 3082 bp genomic fragment representing the Hs1^{pro-1} promoter was isolated from the YAC-DNA housing the Hs1^{pro-1} gene. The promoter analysis with the GUS reporter gene demonstrated that the Hs1^{pro-1} promoter activates a feeding site-specific gene expression in both sugar beet and *Arabidopsis*. Deletion analysis revealed that *cis* elements necessary for nematode feeding site-specific gene expression reside within -355 to $+247$ from the transcription initiation site of Hs1^{pro-1} while upstream elements located between -1199 and -705 are necessary for higher gene expression. To our knowledge, this is the first report on the pathogen responsiveness of a disease resistance gene promoter at the cellular level, thus providing new insight into the gene-for-gene relationship-based plant resistance mechanism.

Materials and methods

Plant material and plant cultivation

The nematode-resistant sugar beet line A906001 carrying the Hs1^{pro-1} gene and the susceptible sugar beet line 93161p were kindly provided by the breeding company A. Dieckmann-Heimburg (Nienstädt, Germany). Plants were grown in a greenhouse at 25 °C. Young leaves were harvested for DNA isolation. For nematode resistance tests in the greenhouse, plants were cultivated in plastic tubes (3 cm $\times$ 4 cm $\times$ 20 cm) filled with silver sand in and nematode resistance tests were performed as described (Toxopeus and Lubberts, 1979). The *A. thaliana* ecotype C24 (Lehle Seeds, TX) was used for plant transformation experiments. *Arabidopsis* plants were cultivated in a growth chamber at 22 °C under a 8 h light/16 h dark cycle.

Plasmid cloning and sequence analysis

The YAC120E7 DNA containing the $Hs1^{pro-1}$ gene was sub-cloned into the plasmid vector pBluescript (Stratagene, USA). Total yeast DNA containing the YAC120E7 was either single- or double-digested with *Bam*HI, *Nco*I, *Sca*I and *Mlu*I and separated on 1% agarose gels.

The restriction pattern of YAC120E7 was determined by Southern analysis with 1832xs as a probe resulting in a ca. 3.4 kb *Nco*I/*Bam*HI fragment covering the upstream region of the $Hs1^{pro-1}$ gene. Hence, fragments ranging in size between 3 and 4 kb were released from the gel by using the gel extraction kit (Qiagen, Hilden, Germany) and ligated to the plasmid vector at 16 °C overnight with T4 DNA-ligase (Promega, Madison, WI). Recombinant plasmids were transformed into *E. coli* DH10b competent cells by electroporation (Gene pulser II, BioRad, Hercules, USA) resulting in a plasmid library with ca. 1650 clones. Bacterial colonies were transferred onto Hybond$^+$ nylon membranes according to the manufacturer's protocol (Amersham-Parmacia, Freiburg, Germany). Colony blots were Southern-hybridized with the 1832xs probe resulting in identification of the clone p2435 which contains the correct upstream sequence of the $Hs1^{pro-1}$ gene (Figure 2). Sequencing of plasmid inserts was performed on a Li-Cor 4000 sequencer following the protocol of Sanger (1977) with the M13 forward/reverse primer IRD800 labelled (MWG, Ebersberg, Germany) and the SequiTherm EXEL II Long-Read DNA Sequencing Kit LC (Epicentre Techn., Madison, WI). Sequences were analysed with LaserGene software (DNASTAR, Madison, WI).

Southern and northern analysis

Genomic DNA was extracted from sugar beet leaves as described by Rogers and Bendich (1985). YAC-DNA was isolated as described by Kleine *et al.* (1995). After restriction, genomic DNA was separated on 0.75% agarose gels and transferred onto Hybond- N^+ membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary diffusion blotting overnight, using 0.25 M NaOH/1.5 M NaCl as blotting solution. Southern blots were hybridized with ^{32}P-labelled DNA probes (Feinberg and Vogelstein, 1983) at 62 °C and washed twice (0.5× SSC, 0.2% w/v SDS) for 30 min and exposed at −70 °C for 48 h.

Four DNA probes were generated by PCR for Southern analysis with plasmid DNA p2435 as a template. 1832yt (1509 bp) was amplified with primers 5′-CATGGTCCAAGACTTATAAC-3′ and 5′-CGATATATTAGTTATTACTACC-3′, 1832lt (419 bp) with primers 5′-GACCTCATCGCTATGTATAAAC-3′ and 5′-AACGATCTTGCTATTCACAGTC-3′, 1832xd (612 bp) with primers 5′-GATTGCAAAACAAAAA-TGGTAC-3′ and 5′-GATTCAACCGCGTAGAAGAT-TC-3′ and 1832xs (398 bp) with primers 5′-GAATTTAGTCTCCTTCCGAG-3′ and 5′-GTAAAT-TCTCGATTTCCTCC-3′. 1832dh (543 bp) representing the codon region of $Hs1^{pro-1}$ was amplified with $Hs1^{pro-1}$-cDNA (GenBank U79733) as template with primers 5′-CGAATAAGTGAGAGGATC-3′ and 5′-GGCACCATCCAAACTCGG-3′. PCR products were separated on 1% agarose gels. Fragments were released from the gel with the gel extraction kit (Qiagen, Hilden, Germany) ready for probe labelling.

Total RNA was extracted from roots of sugar beet using the TRIZOL method (Gibco-BRL, Life Technologies, Karlsruhe, Germany). Total RNA (20 μg) was electrophoresed on 1.3% agarose-formaldehyde gels and transferred onto Hybond-N^+ membrane with 20× SSC. RNA-blots were hybridized with the ^{32}P-labelled 1832dh. The 18S rRNA probe, kindly provided by Dr T. Schmidt (Institute of Crop Science and Plant Breeding, Kiel, Germany), was used as a control for RNA loading. Hybridization of the blots was conducted in the presence of 5× Denhardt's solution, 5× SSPE, 0.2% SDS, 0.05% w/v denatured fish DNA for 16 h at 55 °C overnight. The blots were washed in 5× SSC once and in 2× SSC twice with 0.2% SDS for 30 min at 55 °C, respectively, and subsequently exposed at −70 °C for 72 h. The optical density of northern hybridization signals was measured with the software Quantity One (BioRad, Munich, Germany).

Transformation of Arabidopsis *plants and sugar beet hairy roots*

The susceptible sugar beet line 93161p was used for the generation of transgenic hairy roots. Leaf stalks were sterilized by submerging in calcium hypochloride (5%) for 10 min, followed by repeated washing in sterile double-distilled water. The sterilized leaf stalks were cut into pieces of 2 cm length and incubated with the *A. rhizogenes* suspension as described below. The infected explants were soaked on sterilized filter paper before cultivation on solid 1/2 B5 medium (Gamborg *et al.*, 1968). After 2 days of co-cultivation in the dark, explants were transferred to 1/2 B5 medium containing 400 μg/ml cefotaxime to eliminate *A. rhizogenes*,

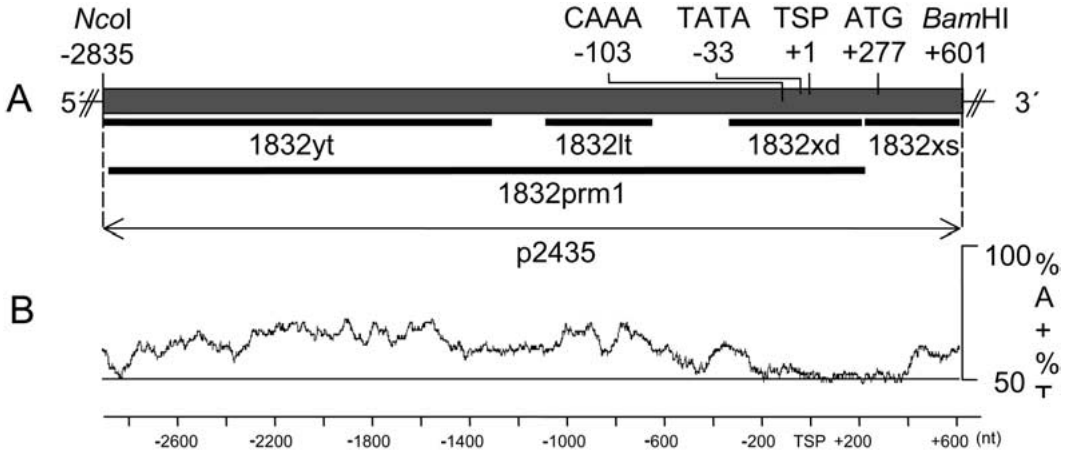


Figure 2. Sequence analysis of the region upstream the Hs1^{pro-1} gene including 601 bp from the transcribed sequence and relative positions of probes and PCR fragments used in this analysis. A. Positions of putative general transcription elements (TATA and CAAT boxes) and the transcriptional (TSP) and translational (ATG) start sites of the Hs1^{pro-1} gene. The positions of genomic fragments generated as probes for further analysis, 1832yt, 1832lt, 1832xd and 1832xs, are shown. B. Relative content as a percentage of the bases adenine and thymine as determined by DNASTAR software.

and incubated at 22 °C under a 16 h light/8 h dark cycle. Single hairy roots 1 cm long were excised and sub-cultured on 1/2 B5 medium containing 150 mg/l cefotaxime under the same conditions.

Arabidopsis transformation with *A. tumefaciens* was conducted as described previously (Bechtholt *et al.*, 1993). The seeds were collected from the infiltrated plants and selected in 1/2 B5 medium containing 100 μg/ml kanamycin. Kanamycin-resistant seedlings were transferred to soil and grown in a growth chamber at 22 °C at a 8 h light/16 h dark photoperiod. Confirmation of the presence of the correct experimental promoter constructs was done in each independent transgenic *Arabidopsis* line and sugar beet hairy root clones by PCR with GUS-specific primers (5′-CCTGTAGAAACCCCAACCCG-3′; 5′-GGTAGATATCACACTCTGTC-3′). T_3-generation of transgenic *Arabidopsis* was used for experiments.

Nematode infection tests and abiotic stress experiments

The nematode culture was kindly supplied by Prof. Grundler, Institute for Plant Pathology, Kiel. Nematodes were propagated on *Sinapis alba* plants grown on 0.2× Knop medium under sterile conditions. Fully developed cysts were removed from the roots onto 50 μm gauze and incubated further with 0.3 mmol $ZnCl_2$ for 8–10 days. Juveniles were harvested with 10 μm gauze and used directly for inoculation experiments. In the greenhouse, 35-day old sugar beet plants, cultivated in plastic tubes filled with silver sand, were inoculated with 2000 infective J2 juveniles as described (Toxopeus and Lubberts, 1979). Inoculation of sugar beet hairy roots and *Arabidopsis* plants with nematodes was performed *in vitro* according to the protocol described by Sijmons *et al.* (1991). Sugar beet hairy roots 1 cm long and 1-week old *Arabidopsis* seedlings were transferred to petri dishes containing 1/2 B5 medium under sterile conditions. One week later, 300 sterile infective J2 juveniles of the beet cyst nematode (*H. schachtii* Schm.) were added to each culture plate. Penetration of nematodes into the roots was observed under a stereomicroscope (Stemi SV 11, Zeiss, Jena, Germany). Nematode inoculation and abiotic stress experiments were repeated 3 times.

For abiotic stress experiments, sugar beet seedlings were grown in the greenhouse as described above. In independent experiments, 5-week old plants were treated by leaf spraying as well as by sand infiltration with 20 ml solutions which contained 100 μM methyljasmonic acid (MeJA), 2 mM salicylic acid (SA), 100 μM abscisic acid (ABA) or 100 μM gibberellic acid (GA). In another experiment, 20 ml 100 mM NaCl solution were directly applied to the roots, and roots were injured with a razor blade repeatedly. At 9 h after treatments, root material was harvested and stored in liquid nitrogen for later isolation of total RNA. The same experiments were also conducted with sugar beet hairy root cultures grown *in vitro*.

For confirmation of responses of plants to the treatments applied in this study, we controlled the tran-

script levels of the PR-1- and the PR-2-like genes as examples in the roots before and after treatments by SA or JA. These two genes had proved to be transcriptionally enhanced in beet roots by treatment with SA and JA, respectively (unpublished data).

Construction of promoter-GUS fusion constructs and Agrobacterium *culture*

For construction of the promoter-GUS fusion constructs, Hs1^{pro-1} promoter fragments covering different regions of the genomic sequence upstream of the transcriptional start site together with 247 bp from the untranslated region of the gene was amplified by PCR (1832prm1, primers 5′-GCACTCGAGATAAGACTCCACCAC-3′ and 5′-CGTAGAAGATTCTAGAAAGCAATC-3′; Δ1.2, 5′-GCTTACTCGAGTGACACTAATCCG-3′ and 5′-GTAGAAGATTCTAGAAGC-3′; Δ0.7, 5′-AAGCCTCTAGACTGTGAATAGC-3′ and 5′-GTAGAAGATTCTAGAAGC-3′; Δ0.5, 5′-TACTCTAGACACCTATATATATAC-3′ and 5′-GTAGAAGATTCTAGAAGC-3′; Δ0.3, 5′-AAACTCAAAAGAAAATTCTAGATT-3′ and 5′-GTAGAAGATTCTAGAAGC-3′) in which *Xho*I and *Xba*I cloning sites were integrated. The PCR fragments were cloned into the pGEM T-vector (Promega) and the insert was confirmed by sequencing. The *Xho*I/*Xba*I fragments and *Xba*I/*Xba*I fragments were released from the T-vector and subcloned into the binary vector pBin19(GUS-Int) upstream of the *gusA* gene resulting in the 1832prm1, Δ1.2, Δ0.7, Δ0.5, Δ0.3 and GUS fusion constructs. The binary vector pBin19(GUS-Int) was constructed by introducing a promoterless GUS intron gene including the 35S terminator, released from the pAm194 plasmid (Planta, Einbeck, Germany), into the *Hin*dIII site of pBin19 (Bevan, 1984). For transformation of sugar beet hairy roots, fusion constructs were electroporated into *A. rhizogenes* strain AR15834 which harbours a wild-type plasmid pRi15834 as described by Kifle *et al.* (1999). For *Arabidopsis* transformation *A. tumefaciens* GV3101 (Koncz and Schell, 1986) was used. The transformed *Agrobacterium* cells were grown overnight on 2YT medium containing 50 mg/l kanamycin and 100 mg/l rifampycin. The overnight cultures were used to inoculate 150 ml 2YT medium without antibiotics and bacteria were cultivated to an OD_{600} of 0.6. The bacterial cultures were centrifuged at 4000 × *g* at 4 °C for 10 min and re-suspended in 15 ml 2YT medium ready for transformation of sugar beet leaf stalks. For *Arabidopsis* transformation by the vacuum infiltration, the overnight-grown *Agrobacterium* cells were grown in 500 ml 2YT medium to an OD_{600} of 1.0. The *Agrobacterium* cells were centrifuged as described above, and then re-suspended in 1 litre infiltration buffer (1/2 MS+50 μl Silwet L-77, Lehle Seeds).

GUS assays

Sugar beet hairy roots and *Arabidopsis* seedlings cultivated on petri dishes were inoculated with nematodes as described above. For histochemical GUS assay 10 ml X-Gluc solution (50 mM NaCl, 100 mM Tris, 100 mM $K_3Fe(CN)_6$, 2 mM X-Gluc (5-bromo-4-chloro-3- indolyl glucuronide, pH 7.0) were added to the petri dishes, and roots were covered by the solution. In this way, the intact structure of feeding sites was maintained. After 16 h of incubation at 37 °C GUS signals were evaluated under a stereomicroscope (Stemi SV 11, Zeiss). The fluorometric GUS assay was performed as described by Jefferson *et al.* (1987). Root tissues (120 mg) were ground with a mortar with 500 μl GUS extraction buffer (50 mM NaH_2PO_4, 10 mm EDTA, 0.1% Triton X-100, 0.1% sarcosyl) and centrifuged at 10 000 × *g* for 10 min at 4 °C. The crude extract of total protein was obtained from the supernatant. The concentration of protein in the supernatant was quantified (Bradford, 1976). GUS activity was measured with 4-methylumbelliferyl-β-D-glucuronide as substrate with the fluorometer DyNA Quant 200 (Pharmacia). The standard curves were prepared with 4-methylumbelliferone. GUS activity was expressed by pmol MU/min per mg protein (Jefferson *et al.*, 1987).

Results

Transcription of Hs1^{pro-1} *is up-regulated upon nematode infection*

The transcript level of the Hs1^{pro-1} gene in resistant beet roots was determined under biotic and abiotic stresses as well as after nematode infection by using RNA gel blot analysis. A time-course study on the Hs1^{pro-1} transcript accumulation after nematode infection was conducted, in which 5-week old sugar beet plants from the nematode-resistant line A906001 carrying the Hs1^{pro-1} gene were inoculated with infectious larvae. Total RNA was isolated from roots harvested 1, 3, 8 and 11 days after nematode inoculation as well as from non-inoculated plants. In addition, total RNA was isolated from roots of sugar beet plants

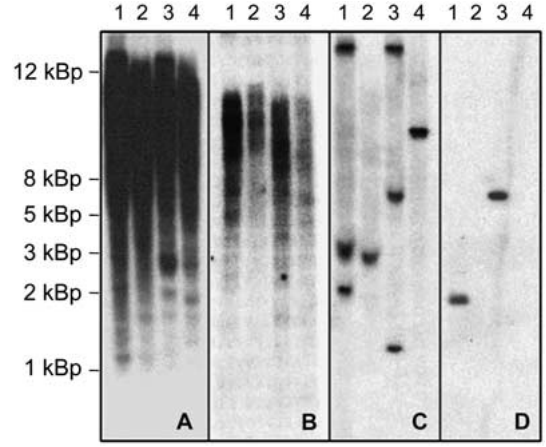


Figure 3. Molecular characterization of the p2435 sequence by genomic DNA blot analysis. A 10 μg portion of DNA of the nematode resistant line A906001 (lanes 1 and 3) as well as of the susceptible line 93161p (lanes 2 and 4) was digested with *Eco*RI (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4), respectively. DNA was separated in 1.0% agarose gels and blotted onto nylon membranes. A. Hybridized with 1832yt. B. Hybridized with 1832lt. C. Hybridized with 1832xd. D. Hybridized with 1832xs. Molecular length markers are indicated at left in kb. Exposure time: 48 h.

which had either been treated with salicylic acid (SA), jasmonic acid (JA), gibberellic acid (GA) and abscisic acid (ABA), wounded with a razor blade or grown under salt stress (NaCl). The RNA gel blots with equal amounts of total RNA were radioactively probed with the Hs1^{pro-1}-specific probe 1832dh and the transcript level of Hs1^{pro-1} was estimated by measuring the optical density of northern hybridization signals. Because our previous results had demonstrated that the Hs1^{pro-1} cDNA represents a wild beet-specific sequence lacking in sugar beet genome (Cai *et al.*, 1997), the susceptible line 93161p was not included in the northern experiment. Figure 1A shows that a 1.6 kb transcript of Hs1^{pro-1} was detected at a low level in roots of the control plants grown in the absence of any stress but increased about fourfold as early as one day after nematode infection. The higher steady-state level of Hs1^{pro-1} mRNA remained until 11 days after inoculation, indicating an enhanced transcriptional activity of Hs1^{pro-1} in the whole infection process. On the other hand, treatments with SA, JA, GA and ABA as well as wounding and salt stress did not result in any effect on Hs1^{pro-1} transcription (Figure 1A) even though responses of plants to treatments applied were confirmed (data not shown). These data strongly suggest *de novo* transcription of Hs1^{pro-1} in resistant beet roots upon nematode infection.

Cloning and Southern analysis of the Hs1^{pro-1} *promoter region*

To clone the sequence upstream of the transcriptional start site of Hs1^{pro-1}, the YAC120E7 from which the Hs1^{pro-1}gene had been isolated (Cai *et al.*, 1997) was shotgun-cloned into a plasmid vector resulting in plasmid p2435. Sequence analysis revealed that plasmid p2435 contains a 3436 bp insertion consisting of 601 bp of the 5′ cDNA sequence of Hs1^{pro-1} and 2835 bp upstream of the transcription start point representing the promoter region of the Hs1^{pro-1} gene (Figure 2). To characterize the cloned genomic sequences, four PCR fragments (Figure 2A) were generated from the p2435 clone, and used as probes for Southern hybridization of genomic DNA from nematode-resistant and -susceptible sugar beet lines. As a result, fragment 1832xs representing a part of the translated region (+192/+590) gave a single-copy signal only with DNA from the resistant line (Figure 3D) as expected. Fragment 1832xd (−399/+273) representing the 5′ region proximal to the transcriptional start site resulted in low-copy signals polymorphic between resistant and susceptible sugar beet lines (Figure 3C) and two PCR fragments representing the distal regions, 1832lt (−1099/−680) and 1832yt (−2835/−1326), respectively, showed hybridization patterns typical of highly repetitive sequences with DNA from both resistant and susceptible lines (Figure 3A, B).

Database-assisted sequence analysis of the Hs1^{pro-1} *promoter region*

The p2435 sequences were analysed using various database search programs including PLACE (http://www.dna.affrc.go.jp; Higo *et al.*, 1999), CISTER (http://zlab.bu.edu; Frith *et al.*, 2001) and PlantCARE (http://sphinx.rug.ac.be:8080/PlantCARE; Lescot *et al.*, 2002) to identify general transcription and potential regulatory elements. The A/T content of the upstream sequence relative to the transcriptional start site of Hs1^{pro-1} is up to 68.5% (Figure 2B). A putative TATA box is localized 33 bp upstream from the transcriptional start site (Figure 2A) with the core sequence gTACATAAAccgg which significantly matches the consensus TATA box motif (g/ctA/tTA/tA/TAa/tg/ag/cc/gg/cg/t) and the average distance between TATA box and transcription start site (Bucher, 1990). The same core sequence TACATAAA had also been identified in TATA box of rice T42 gene promoter that triggers stamen-specific gene expression (patent WO9213956-A/7). A putative CAAT

box displays at position −103 with the sequence TCAAAC.

Several *cis* elements were identified in the Hs1^{pro-1} promoter region, which are similar to those previously described in defence-related and elicitor-responsive genes (Figure 4). A 12 bp imperfect repeat with the core sequence CAATTG is present between nucleotides −1906 and −1855. A nearly identical element had been reported as putative transcription factor-binding domain of the *LEMMI9* promoter up-regulating *LEMMI9* expression in giant cells of tomato plants upon root knot nematodes (Escobar *et al.*, 1999). The region between −954 and −885 share 91% similarity with the proximal region of the *Dgrca* (*Datisca glomerata)* Rubisco activase promoter that activates the tissue-specific expression of *Dgrca* in symbiotic root nodules of the actinorhizal plant *D. glomerata* after infection with the actinorhizal species *Frankia* (Okubara *et al.*, 1999). In addition, two root-specific elements (RSEs) with the core sequence CAACTTT that are highly conserved in promoters of several root-specific genes (Keller and Heierli, 1994; Elmayan and Tepfer, 1995; Elliott and Shirsat, 1998) were found between −795 and −789 as well as between −399 and −393. Two TTGACC (W box) motifs appear in the region between −999 and −876 representing a potential recognition site for plant WRKY-DNA-binding proteins. W boxes had proved to be a major class of *cis*-acting elements essential for elicitor-responsiveness (Eulgem *et al.*, 1999, 2000; Yu *et al.*, 2001). In addition, the elements CCGTCC at −929/−935 and TCTCACCTATCT at −389/391 significantly match Box A (CCGTCC) and Box L (t/cCt/cc/tACCt/aACC), respectively, which are involved in elicitor- and UV irradiation-mediated PAL gene activation (Lois *et al.*, 1989; Logemann *et al.*, 1995). Also several potential binding sites for plant Myb transcription factors are found in the Hs1^{pro-1} promoter region indicating possible roles of Myb-related proteins in regulation of the Hs1^{pro-1} expression as that found in several plant stress-related genes (Urato *et al.*, 1993; Rushton and Somssich, 1998; Yang *et al.*, 2001). It is interesting to note that a perfect CACGTG palindrome sequence corresponding to the G box was found in duplicate copies at +61 and +218, together with a as-1 element (TGACGTCA) in the untranslated region representing possible binding sites for bZIP transcription factors (Pla *et al.*, 1993; Yamagochi-Shinozaki and Schinozaki, 1994; Ishige *et al.*, 1999; Guan *et al.*, 2000).

Functional analysis of the Hs1^{pro-1} *promoter region in sugar beet roots*

To define the regulatory activity of the Hs1^{pro-1} promoter sequence, the genomic fragment 1832prm1 containing 2835 bp sequence upstream of the transcriptional start site and 247 bp 5′-UTR of the Hs1^{pro-1} gene was generated by PCR. Due to a strong autofluorescence of sugar beet roots, GFP (green fluorescent protein) was not suitable as a reporter gene for this analysis. Instead, 1832prm1 was fused to the promoterless β-glucuronidase reporter gene in the binary vector pBIN19(GUS-Int) resulting in the construct 1832prm1::GUS and subsequently introduced into hairy roots of the susceptible beet line 93161p by *Agrobacterium rhizogenes*-mediated transformation (Kifle *et al.*, 1999). Line 93161p had been successfully used for functional analysis of the Hs1^{pro-1} gene (Cai *et al.*, 1997) and proved to be easily transformed, compared to the nematode-resistant line A906001. In total, 39 independent hairy root clones were obtained, 22 of which gave a clear PCR product with GUS gene-specific primers (data not shown). These 22 PCR-positive root cultures were employed subsequently for GUS activity assays, together with 20 roots transformed with the pBin19(GUS-Int) vector carrying the promoterless GUS gene, which served as control. Responses of 1832prm1::GUS transgenic roots to nematode infection or abiotic stress factors were analysed *in vitro*. For determining response to nematode infection, each root clone was inoculated with 300 infectious nematode juveniles and GUS activity was measured 11 days later. In the absence of infectious nematodes, weak GUS activities could be detected in transgenic roots carrying the 1832prm1::GUS construct but it was significantly higher than the background measured in control roots carrying the promoterless GUS gene (Figure 5), indicating a basal promoter activity in consistence with the RNA blot analysis results. After nematode infection, GUS activities significantly increased in 19 of 22 selected transgenic roots carrying the 1832prm1::GUS construct (Figure 5) whereas no change in GUS activity was observed in control roots carrying the pBin19(GUS-Int) vector. In addition, treatments with SA, JA, GA, ABA or wounding and salt stress (NaCl) did not result in changes in the GUS activities either in the 1832prm1::GUS-transgenic roots or in control roots. These results are in agreement with those observed from the RNA-gel blot analysis and indicate that the 1832prm1 sequence contains all the pro-

Figure 4. DNA sequenec of the Hs1^{pro-1} promoter region. Numbering starts at the transcription start site (TSP, +1). The transcription start site is marked by arrowhead. The translation start site (ATG) and the putative TATA box are in bold. DNA regions representing potential *cis* elements discussed in the text are boxed. *Asterisks* indicate identical nucleotides compared to the known *cis* elements found in promoters of defence-related and elicitor-responsive genes: *LEMMI9*, CAATTG (Escobar *et al.*, 1999); W box, (T)TGAC(C/T) (Rushton *et al.*, 1996; Yu *et al.*, 2001); RSE, CAACTTT (Keller and Heierli, 1994); G box, GCCACGTGCC (Ishige *et al.*, 1999); Box A, CCGTCC; Box L, (T/C)C(T/C)(C/T)ACC(T/A)ACC (Logemann *et al.*, 1995); ACIof PAL-2, CCCACCTACC (Hatton *et al.*, 1995); As-1, TGACGTCA (Yamagochi-Shinozaki and Schinozaki, 1994); Myb, CNGTTR, GKTWGTTR, GKTWGGTR, GGATA (Romero *et al.*, 1998; Baranowskij *et al.*, 1994); Myc, CACATG (Abe *et al.*, 1997). DNA region showing homology with Rubisco activase is underlined (Okubara *et al.*, 1999).

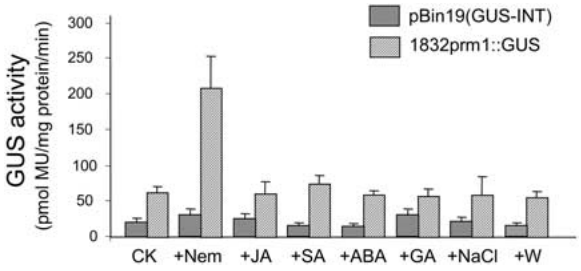


Figure 5. GUS activity in 1832prm1::GUS sugar beet hairy roots under biotic and abiotic stress as well as after nematode infection. Roots transformed with pBin19(GUS) served as control. CK, untreated hairy roots; Nem., hairy roots inoculated with nematodes 7 days after infection; JA, hairy roots treated with 100 μM jasmonic acid; SA, hairy roots treated with 2 mM salicylic acid; ABA, hairy roots treated with 100 μM abscisic acid; GA, hairy roots treated with 100 μM gibberellic acid; W, hairy roots wounded with a razor blade. The GUS activity level is given as mean values (column) of 3 independent measurements of 19 transgenic root clones. Error bars equal half the standard deviation.

moter elements necessary for activation of nematode-responsive gene expression in sugar beet.

To elucidate the regulation of the Hs1^{pro-1} gene expression on the cellular level, the GUS expression pattern of 1832prm1::GUS transgenic roots was analysed after nematode infection by a histochemical GUS assay. No any GUS signal was observed either in the 1832prm1::GUS transgenic roots or in the control roots without nematode infection and a basal promoter activity could not be detected in this way (data not shown). Strong GUS signals appeared in 1832prm1::GUS-transgenic roots 11 days after nematode infection, which were all restricted to the nematode feeding sites that later develop into syncytia (Figure 6B and C). No GUS staining was detectable in the control roots (Figure 6A). A time-course observation with the 1832prm1::GUS transgenic roots that were nematode-inoculated between 1 and 11 days after infection revealed that GUS expression started early (1 day after infection) with formation of the initial syncytial cell (ISC).

Not all feeding sites/syncytia in the 1832prm1::GUS transgenic roots showed the GUS staining (Table 1). Strong variations in frequency of the GUS-stained syncytia were observed among root clones, which ranged between 20% and 70% of all observed feeding sites (Table 1). These variations were obviously due to the testing system in which only the feeding sites that developed in close vicinity to the surface of the B5 medium in the root culture plate were stainable with the GUS incubation buffer. Control roots carrying the pBin19(GUS-Int) vector were analysed in the same manner but no GUS signals were visible either in nematode feeding sites or in other root cells. Moreover, wounding and application of NaCl or treatment with ABA, GA or JA did not result in any GUS staining in feeding sites of the 1832prm1::GUS transgenic roots.

Deletion analysis of the Hs1^{pro-1} *promoter region*

To characterize the Hs1^{pro-1} promoter and define *cis* elements responsible for feeding site-specific gene expression, four fusion genes were constructed with various 5′-deletion fragments of the Hs1^{pro-1} promoter (1832prm1) and the β-glucuronidase gene in the binary vector pBin19(GUS-Int) (Figure 7). The resulting constructs (Δ1.2, Δ0.7, Δ0.5 and Δ0.3) were transferred into sugar beet hairy roots via *Agrobacterium*-mediated transformation. More than 10 independent transgenic hairy roots for each construct were selected and used for further GUS activity assays. Roots transformed with the construct 1832prm1::GUS and the pBin19(GUS-Int) served as controls.

GUS activities of transgenic roots carrying various constructs were fluorimetrically measured 11 days after nematode inoculation. Figure 7 shows that the Hs1^{pro-1} promoter 5′-deleted up to the -1199 (Δ1.2) retained the promoter activity as high as the full-length promoter. However, a further deletion to -705 resulted in a drastic reduction of the promoter activity, roughly to 40% of that of the full-length promoter. Sequential deletions to -487 or to -355 (Δ0.5 and Δ0.3) did not cause further decrease in the GUS activity any more. By comparison, no significant changes of GUS activities in untreated roots were observed as indicated by the ratio values (Figure 7). Histochemical GUS staining revealed that the GUS signals appeared consistently only in feeding sites of transgenic roots carrying each deletion construct including those with the Δ0.3 construct. Even by visual observation a strong reduction of GUS-staining intensity was obvious in feeding sites of transgenic roots carrying the Δ0.3, Δ0.5 or Δ0.7 constructs, compared to those transformed with the Δ1.2 or the 1832prm1::GUS constructs. These data, taken together, suggest that the deletion fragment ($-1199/+247$) contains essential promoter and enhancer elements for activation of Hs1^{pro-1} expression in beet roots. One enhancer element is located between -1199 and -705 while *cis*-acting sequences responsible for feeding site-specific expression of Hs1^{pro-1} residue within the promoter region between -355 and $+247$ represented by the Δ0.3 construct.

Table 1. Results from histochemical GUS-staining of syncytia established in transgenic sugar beet hairy roots and transgenic *Arabidopsis* plants 11 days after nematode infection.

Construct	Transgenic sugar beet hairy roots		Transgenic *Arabidopsis* plants	
	number of syncytia established	number of GUS stained syncytia	number of syncytia established	number of GUS-stained syncytia
1832prm1::GUS	148	73	119	77
pBin(GUS-INT)	98	0	84	0

Functional analysis of the $Hs1^{pro-1}$ *promoter in* Arabidopsis *plants*

To prove the nematode-responsiveness of the $Hs1^{pro-1}$ promoter in whole plants and against a different genetic background, the constructs 1832prm1::GUS and pBin19(GUS-Int) that served as a control were transformed into *Arabidopsis* by using the vacuum infiltration procedure. In total, 11 transgenic lines carrying the 1832prm1::GUS construct and 6 control lines were obtained. For further analysis, 4 different lines were selected which carried the 1832prm1::GUS construct as determined by Southern analysis with the GUS codon sequence as a probe (data not shown). For microscopic observation, the *Arabidopsis* seedlings were grown under sterile conditions on petri dishes containing the B5 medium.

For each line, five plates each containing five *Arabidopsis* seedlings were inoculated with nematode juveniles. At 11 days after nematode inoculation, histochemical staining for determining GUS activity was performed in root, leaf and also hypocotyl of both 1832prm1::GUS and pBin19(GUS-Int) seedlings. Nearly the same GUS expression pattern in root tissues of the 1832prm1::GUS transformants was observed as compared to sugar beet root hairy roots. GUS signals appeared early in the ISCs and were later restricted to syncytia of the 1832prm1::GUS roots (Figure 6E). No GUS staining was detectable in the control plants (Figure 6D). As described above for sugar beet hairy roots, not all syncytia in the 1832prm1::GUS *Arabidopsis* transformants exhibited GUS staining. At 11 days after nematode inoculation, 77 out of 119 syncytia investigated on 1832prm1::GUS transformants (65%) exhibited GUS staining (Table 1). In addition, histochemical staining of leaves, stem and reproductive tissues of 1832prm1::GUS transformants did not reveal any GUS signals (data not shown) with the exception of weak GUS staining in hypocotyl tissue of some 1832prm1::GUS transformants (Figure 6F) after nematode infection.

Discussion

In this study, the transcriptional regulation of the $Hs1^{pro-1}$ gene was studied in response to various stress applications and the $Hs1^{pro-1}$ transcriptional activity was investigated with promoter-GUS fusion constructs on the cellular level. The main results can be summarized as follows. First, *de novo* transcription of the $Hs1^{pro-1}$ gene was induced after nematode infection; second, the transcriptional activation of the $Hs1^{pro-1}$ promoter was restricted to nematode feeding sites. *Cis* elements responsible for feeding site-specific gene expression reside between -355 and $+247$ and an enhancer region is located between -1199 and -705 from the transcriptional initiation site of $Hs1^{pro-1}$; third, abiotic stress and plant signal molecules did not activate the $Hs1^{pro-1}$ transcription; and fourth, the nematode responsiveness of the $Hs1^{pro-1}$ promoter was independent from the genetic background demonstrating the generality of this response.

Most plant disease resistance genes cloned so far have been reported to be expressed constitutively like the two nematode resistance genes *Mi* (Milligan *et al.*, 1998) and *Gpa2* (van der Vossen *et al.*, 2000). Only two resistance genes were reported to be up-regulated either upon pathogen infection or by environmental stress or treatment with certain chemicals. The first one is *Xa1*, a bacterial resistance gene from rice (*Oryza sativa*), which is up-regulated after infection with *Xanthomonas oryzae* pv. *oryzae* (Yoshimura *et al.*, 1998). The second one, the *pib* rice blast resistance gene, is up-regulated under environmental conditions favouring pathogen infection and by chemical signals that trigger secondary plant resistance (Wang *et al.*, 1999, 2001). As revealed by RNA-blot analysis in this study, the $Hs1^{pro-1}$ gene is expressed

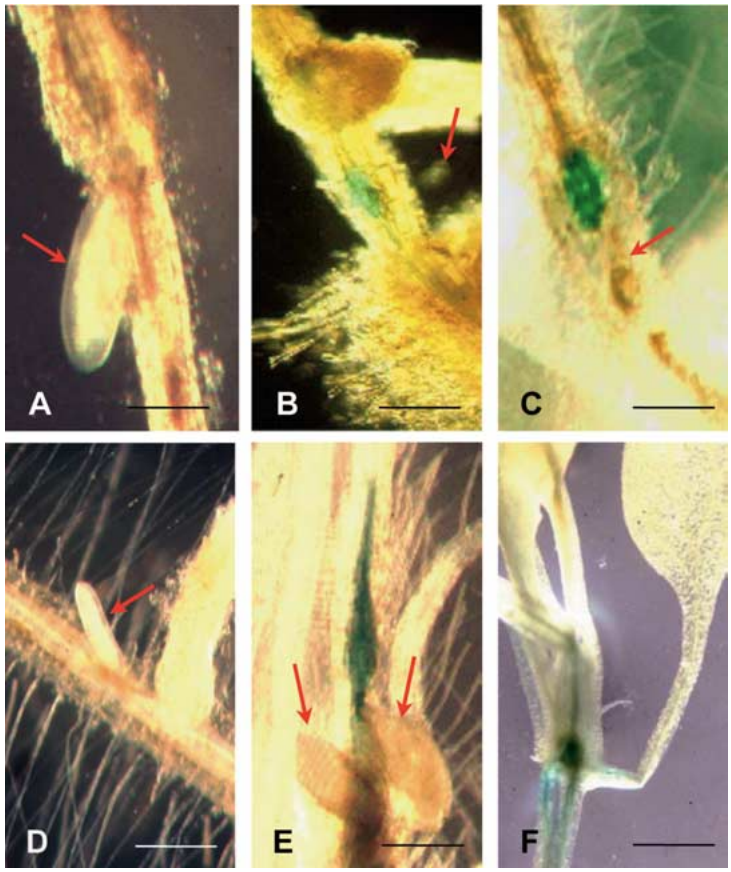
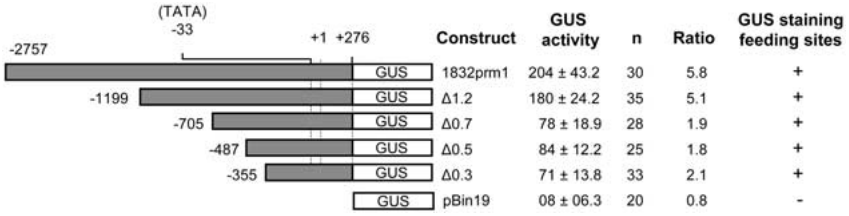


Figure 6. Histochemical staining of GUS activity of 1832prm1::GUS-transgenic sugar beet hairy roots and *Arabidopsis* plants after nematode infection 11 days after infection. A. Hairy roots transformed only with pBin19(GUS-Int) without any GUS staining. B and C. Hairy roots transformed with 1832prm1::GUS showing GUS staining only within the feeding structure. D. Roots of *Arabidopsis* plants transformed with pBin19(GUS-Int) without any GUS staining. E. Roots of *Arabidopsis* plants transformed with 1832prm1::GUS showing strong GUS staining within the syncytium. F. Hypocotyl tissue of *Arabidopsis* plants transformed with 1832prm1::GUS revealing weak GUS activity. Nematodes are marked by red arrows. The bars equal 250 μm (A and B), 200 μm (C), 500μm (D and E) or 100 μm (F).



Construct	GUS activity	n	Ratio	GUS staining feeding sites
1832prm1	204 ± 43.2	30	5.8	+
Δ1.2	180 ± 24.2	35	5.1	+
Δ0.7	78 ± 18.9	28	1.9	+
Δ0.5	84 ± 12.2	25	1.8	+
Δ0.3	71 ± 13.8	33	2.1	+
pBin19	08 ± 06.3	20	0.8	-

Figure 7. Structure of GUS fusion constructs and determination of GUS activity in transgenic sugar beet hairy roots. The shaded boxes represent various deletion fragments of the *Hs1pro-1* promoter (Δ1.2, Δ0.7, Δ0.5 and Δ0,3). Numering starts with the transcription start site (+1). The putative TATA box is indicated. GUS activities presented are the mean values (pmol MU per mg protein per minute) of (*n*) individual roots including SD (±) and the ratio of GUS activities between infected and uninfected roots. The GUS assay was repeated three times. Stained (+) and unstained (−) feeding sites are indicated.

at a low level in uninfected sugar beet roots. It can be speculated that the low activity of the Hs1^{pro-1} gene is required for recognition of the parasite to initiate resistance responses. Upon nematode infection, expression of the Hs1^{pro-1} gene was locally induced. Transcriptional activity reached its maximum as early as one day after nematode infection. These results demonstrate that enhanced expression of Hs1^{pro-1} in feeding structures might be crucial for resistance. Studying Pto and NPR1 recently demonstrated the importance of enhanced expression of *R* genes for plant disease defence responses. The tomato disease resistance gene *Pto* specifies race-specific resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. But, over-expression of *Pto* in tomato activates defence responses in the absence of the Pto-AvrPto interaction and confers broad disease resistance (Tang *et al.*, 1999). *NPR1*, a positive regulator of inducible plant disease resistance in *Arabidopsis*, is up-regulated by pathogen infection or treatment with defence-inducing components such as salicylic acid (Zhang *et al.*, 1999; Després *et al.*, 2000; Kinkema *et al.*, 2000; Yu *et al.*, 2001). Upon pathogen infection the expression of *NPR1* is two- to threefold enhanced facilitating the activity of specific transcription factors activating the transcription of *PR-1* genes, which ultimately results in disease resistance (Eulgem *et al.*, 1999). Overexpression of *NPR1* in *Arabidopsis* leads to enhanced resistance to both *P. syringae* and *Peronospora parasitica* in a dosage-dependent fashion (Cao *et al.*, 1998).

Transcriptional activation of the Hs1^{pro-1} gene may increase the amount of Hs1^{pro-1} protein within feeding cells, a critical factor for induction of plant resistance responses. This is supported by the cytological observation on roots of conventionally bred sugar beet carrying the Hs1^{pro-1} gene under the control of its own promoter. The J2 juveniles were able to invade the roots and proceed to the vascular cylinder to initiate formation of the ISC. However, syncytia did not develop regularly due to the formation of distinct membrane aggregations filling large parts of the syncytium, consequently causing the degradation of the syncytia at early developmental stages and the death of the nematode juveniles (Holtmann *et al.*, 2000). Thus, exploring the role of the Hs1^{pro-1} protein in the formation of this aggregations within the syncytia is likely a key step towards understanding the Hs1^{pro-1}-mediated nematode resistance in plants. The various processes of response of tomato after infection with *M. incognita* were described by Paulson and Webster (1972). They observed the formation of necrotic tissue only 12 h after penetration of the nematodes. This immediate reaction of the plant seems to be a common phenomenon of resistance against sedentary nematode species (Rice *et al.*, 1987; Grundler *et al.*, 1997).

In general, induced expression of genes in nematode feeding sites is found to be required for establishment of the feeding structures by nematodes. Also, reports have been given about promoters of several plant genes to be responsive to nematode infection (Cramer, 1992; Goddijn *et al.*, 1993; Opperman *et al.*, 1994; Niebel *et al.*, 1996; Fenoll *et al.*, 1997; Puzio *et al.*, 1998; Escobar *et al.*, 1999). During a compatible plant-nematode interaction, the *LEMMI9* gene is up-regulated in giant cells of tomato plants by the root-knot nematode (Van der Eycken *et al.*, 1996). The regulatory elements responsible for this up-regulation were mapped on a 111 bp length genomic fragment upstream of the transcription start point, where a 12 bp direct imperfect repeat with the core sequence CANNTG is probably responsible for the nematode responsiveness. It will be interesting to find out the mechanism of gene activation upon nematode infection, which distinguishes compatible from incompatible host-nematode interactions.

Cis-acting sequences regulating eukaryotic gene expression are often combinatorial (Benfey *et al.*, 1989). Consistently, several *cis*-elements were predicted by various database search programs in the Hs1^{pro-1} promoter region, which are similar to those previously described in defence-related and elicitor-responsive genes. Southern analysis demonstrated that the Hs1^{pro-1} promoter sequence, other than the Hs1^{pro-1} coding region, does not show wild beet specificity. These data strongly indicate some general regulatory elements conserved in the plant kingdom are involved in regulation of Hs1^{pro-1} expression. Furthermore, the Hs1^{pro-1} promoter is enriched with repetitive DNA elements. The frequency of those elements was increased with the distance from the transcriptional start point as revealed by the complexity of the hybridization signals. Repetitive motifs can function as regulatory elements or alter the physical distance between *cis* elements and affect transcriptional processes (White *et al.*, 1994). Moreover, these elements may enhance recombination frequencies as part of an evolutionary procedure to establish new plant-pathogen interactions (Michelmore *et al.*, 1998). It remains a great challenge to find out the role of these elements in regulation of Hs1^{pro-1} expression.

Several lines of evidence indicate that the whole regulatory region of the Hs1^{pro-1} gene has been cloned. The 5′-flanking sequence has a high content of adenine and thymine bases typically for regulatory sequences. The A+T content decreases 200 bp upstream of the transcription start site. Various putative regulatory *cis* elements for biotic and abiotic stress responses were found in the 5′-flanking region between −1199 and +247 relative to the transcriptional start point. Probably, some of these motifs are part of a general regulatory machinery leading to responsiveness of plant genes to biotic and abiotic stress. Deletion from −1199 to −705 resulted in significant reduction of GUS activity but not expression pattern in transgenic roots indicated that a possible enhancer region had been removed. Examination of the deleted sequences did not identify potential enhancer *cis*-acting elements except for several CAAT-like boxes, two W boxes and an A-box motif of the PAL promoter. Thus, to define enhancer elements within this region, further 'narrow-down' experiments are required. The fact that sequential deletions to −355 did not cause changes of the GUS expression pattern in transgenic roots gave the evidence that *cis*-acting sequences necessary for the feeding site-specific expression reside between −355 and +247 relative to the transcription initiation site of Hs1^{pro-1}. The expression profile of the GUS reporter gene correlates with those observed in northern analysis, implying that GUS activity profiles are accurate reporters of Hs1^{pro-1} gene expression. Several *cis*-acting elements were identified between −355 and +247. Most strikingly, two G-box-like elements, together with an as-1 element, are found in the non-translated region of the Hs1^{pro-1} gene. These elements interacting with bZIP transcription factors (Pla *et al.*, 1993; Guan *et al.*, 2000) are found to be present in many plant genes that are regulated by a variety of environmental, physiological signals and plant defenses (reviewed in Rushton and Somssich, 1998). The absence of significant changes in GUS activities in untreated roots carrying various deletion constructs suggests that repressor elements may not be involved in the Hs1^{pro-1} gene regulation.

It is important to note that homology to a previously described nematode-responsive promoter motif could be found within the Hs1^{pro-1} promoter although the *LEMMI9* promoter element from tomato is responsive to a different nematode species, *M. incognita*. It will be interesting to find out whether two unrelated plants use the same mechanism of gene activation upon infection of different genera of nematodes although *LEMMI9* is part of the compatible host-nematode interaction. The sequence similarity to the promoter of the Rubisco activase gene from spinach (*Spinacia oleracea*) (Werneke *et al.*, 1989) may give another hint for specific regulatory activity. The Rubisco activase gene is present in all higher plants, predominantly expressed in green tissue (Liu *et al.*, 1996). In *D. glomerata* the Rubisco activase *Dgrca* is specifically up-egulated in symbiotic root nodules after infection with the actinorhizal species *Frankia* (Okubara *et al.*, 1999). This may present a first evidence for a general mechanism of gene activation in highly specialized root cells, which undergo drastic changes in metabolism and cell function after infection with either symbiotic or pathogenic micro-organisms.

Since *Arabidopsis* is also a host for the beet cyst nematode (Sijmons *et al.*, 1991), the promoter activity of the cloned genomic sequence upstream from the transcriptional start site of Hs1^{pro-1} was tested in transgenic *Arabidopsis* plants, which provided an ideal model system to study plant-nematode interaction. The *Arabidopsis* experiments clearly indicate that the Hs1^{pro-1} promoter is active in an unrelated plant. The expression pattern revealed by histochemical staining in *Arabidopsis* is consistent with that in sugar beet hairy roots. This indicated that the signalling pathways leading to transcriptional activation of Hs1^{pro-1} is highly conserved between these two species. In rare cases, weak GUS staining appeared in hypocotyl tissue reflecting probably ectopic expression in heterologous promoter system. Most importantly, activity was only detectable after nematode infection and restricted to feeding sites as in sugar beet, thus confirming the nature of nematode-responsiveness of the Hs1^{pro-1} promoter. It is known that a large number of transcriptional processes are changed in the course of the nematode infection process (Barthels *et al.*, 1997), which could be triggered by the nematode either in a direct or in an indirect manner. In the second case, the nematode may activate enzymatic processes within the feeding cells, which eventually result in the change of activity of specific transcription factors that bind to the Hs1^{pro-1} promoter elements. In this context, conserved transcription factors have to be present in both sugar beet and *Arabidopsis*. Alternatively, a factor delivered by the nematode into the cell via its stylet functions as a transcription factor activating the Hs1^{pro-1} gene. This hypothesis could be supported by the fact that proteins derived from the subventral gland of the nematode are active during parasitism within the root (Rosso *et al.*,

1999; Davis *et al.*, 2000; Popeijus *et al.*, 2000; Goellner *et al.*, 2001). However, no evidence has been given that a nematode factor can be released into a nucleus of the feeding cells for direct gene activation. The direct interaction between a pathogen factor and a resistance gene promoter would be a special aspect of the elicitor-receptor concept, because so far the molecular plant-pathogen interaction is understood as an interaction between proteins of the pathogen and of the host either within or outside the cell (Flor, 1971; Martin *et al.*, 1993).

Independent of the nature of the interaction between plant and parasite, the $Hs1^{pro-1}$ promoter described here has a potential for engineering nematode resistance in plants (Jung *et al.*, 1998). Because it is exclusively enhanced in feeding sites in response to nematode infection the responsive element can be used for controlling a lethal gene, such as the Rnase gene from *Bacillus amyloliquefaciens* (Mariani *et al.*, 1992) whose expression results in rapid cell death, consequently leading to death of the nematode juveniles shortly after initiation of the feeding structure.

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